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**13. ABSTRACT** *(Maximum 200 Words)*
Loss of neurons due to neurotoxin exposure result in various forms of neurodegenerative disorders (ND). Promising strategies include transplantation of donor cells. The quantity and quality of donor cells however has been a problem. We have been exploring DNA molecular decoy as a way to engineer donor cells that are suitable for transplantation. We have been using a double stranded (ds) DNA molecule that includes the septamer motif (Dobi et al., 2000). According to Objective #1: we have determined that a) the optimal developmental stage for decoy using the rat brain is between E16 and E20; b) the duration of 4 days of decoy is sufficient for generating BrdU+/nestin+ cells; c) the decoy molecule is sufficiently stabile without additional modifications, d) using DNA delivery systems (PEI or DOTAP) for increased delivery of dsDNA decoy molecules increased toxicity. In addition, we have determined that the septamer decoy molecule specifically interact with the septamer nuclear complex without interfering with other DNA binding regulatory proteins; established a fast and easy assay system for testing decoy molecules; determined that septamer DNA decoy decreases the number of postmitotic (MAP2+) neurons, suggesting a potential "dedifferentiation". The differentiation potential of decoyed cells (Objective #2) are to be tested in the next set of experiments.

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**Introduction:**

Neurotoxin exposure often results in significant loss of neurons and leading to neurodegenerative diseases (NDs). One the most promising strategy for restoring functionality in NDs is the cell substitution therapy (neuronal transplantation). The success of the neuronal transplantation however is greatly dependent on the quantity and quality of donor cells. Ideal donor cells are the multipotent stem cells or neuronal progenitors. However, obtaining sufficient numbers of cells in a progenitor stage has been challenging. Attempts have included immortalizing primary cells by oncogenic transformation, expanding primary multipotent embryonic stem cells/multipotent progenitors by using cocktails of soluble factors. The main problem using these factors is that generating sufficient quantity of cells requires substantial amount of time.

We have identified a family of nuclear regulatory proteins (septamer DNA binding proteins, Dobi et al., 2000) that controls neuronal (and glial) differentiation. In our application we have prposed to use DNA molecular decoy technology to alter the function of septamer proteins and use the technique to engineer cells for transplantation.

**Body:**

Objective #1: To optimize DNA molecular decoy:

Experiment #1. To determine the optimal developmental stage of cells for septamer DNA decoy.

Results: We have tested cell cultures derived from striatum and cortex at developmental stages of E16 and E20.

Following septamer DNA molecular decoy, we have observed the expected increase in the numbers of progenitor (BrdU+/nestin+) cells. We also observed a substantial decrease in the number of postmitotic differentiating neurons (MAP2+ cells) (Fig. 1).

Conclusions: Based on these observations, we will not need to use cultures derived from brain regions that yield very small quantity of cells (e.g. ganglionic eminences). Apparently decoy works as long as there are late dividing or early postmitotic cells present. This will broaden the usage of original tissues/cells to be decoyed.

Experiment #2. To determine the optimal duration of DNA decoy.

Results: Decoying cells for 3 days resulted substantial increase in the number of progenitor (BrdU+/nestin+) cells. We will perform the longer exposures in conjunction with Objective #2 (characterization of the developmental potential of decoyed cells).

Conclusions: Short decoy (3 days) can result in a sufficient increase in progenitor cell numbers. Longer exposure to decoy molecules and extended culturing can induce additional changes, so we will take the conservative approach and use cells for Objective #2 derived from short decoyed cultures.

Experiment #3. To optimize the intracellular stability of decoy DNA molecule.

Results: Experiments #1 and #2 have shown that the concatamerized dsDNA containing the septamer motif is sufficiently stabile in altering the cellular phenotype. We have calculated the additional costs of introducing

chemical modifications would double the cost of the dsDNA decoy molecule.

Conclusions: Additional chemical modifications would be prohibitively expensive to generate without any potential promise for better results thus we have discontinued these experiments.

Experiment #3a. To test the specificity septamer decoy DNA molecule.

Results: Because of the close similarities of septamer DNA element to other known DNA motifs we have tested the specificity of septamer DNA binding using sets of mutations (Fig. 2).

Conclusions: Septamer DNA decoy molecule is highly specific and does not interfere with any other known octamer or POU proteins.

Experiment #3b. To establish a rapid and inexpensive system for testing decoy DNA molecules.

Results: Generating sufficient quantity of septamer dsDNA decoy molecule is expensive because we have been using concatamerized (multiple copies) of the septamer sequence. (Current length is 72 nucleotides). To make septamer DNA decoy more cost effective we will test the minimum length of dsDNA required for biological effect. To that end we have developed a rapid and assay system based on competitive transfection (Fig. 3).

Conclusions: The competitive transfection using a reporter gene enables us to test various truncated septamer dsDNA decoy molecules so we can identify the shortest (least expensive) molecule.

**Key research accomplishments:**

1) Broadened range of tissues/cells (not restricted to early embryonic brain) can be successfully decoyed using septamer dsDNA.
2) Short decoy (3 days) is sufficient for successfully altering the cellular phenotype.
3) Septamer DNA decoy molecule is highly specific and does not interfere with any other nuclear regulatory pathways.
4) A rapid and inexpensive assay system for testing shortened (less expensive) decoy DNA molecules is developed and successfully tested.

**Reportable outcomes:**

A.L. Dobi and D. v. Agoston (2002) A novel clone selection procedure for expression cloning.
Biotechniques (in press)

A. Dobi, W. Debnam, and D. v. Agoston (2002) A novel transcriptional regulator of astroglia differentiation; the heterogeneous nuclear ribonucleoprotein (hnRNP) A2B1.
Ninth USUHS Research Day, Bethesda, MD

Denes v. Agoston, Albert Dobi, Francis Lim, Miklos Palkovits, Mary Ring, Marianna Szemes, (2002) DNA AND PROTEIN COMPONENTS OF THE NUCLEAR REGULATORY CODE DURING NEURAL DIFFERENTIATION.
Cold Spring Harbor Symposium: Dynamic Organization of Nuclear Function, Cold Spring Harbor, NY

**Conclusions:** Septamer DNA molecular decoy can be used efficiently for altering the differentiation stage of late mitotic - early differentiating neurons. The differentiating potential of decoyed cells will be established as planned in Objective #2. Current efforts also include determining the minimal molecular requirement for effective (reducing the length of the decoy molecule) that will be important before large-scale use.

**References: none**

**Appendices: Figures 1 through 3.**

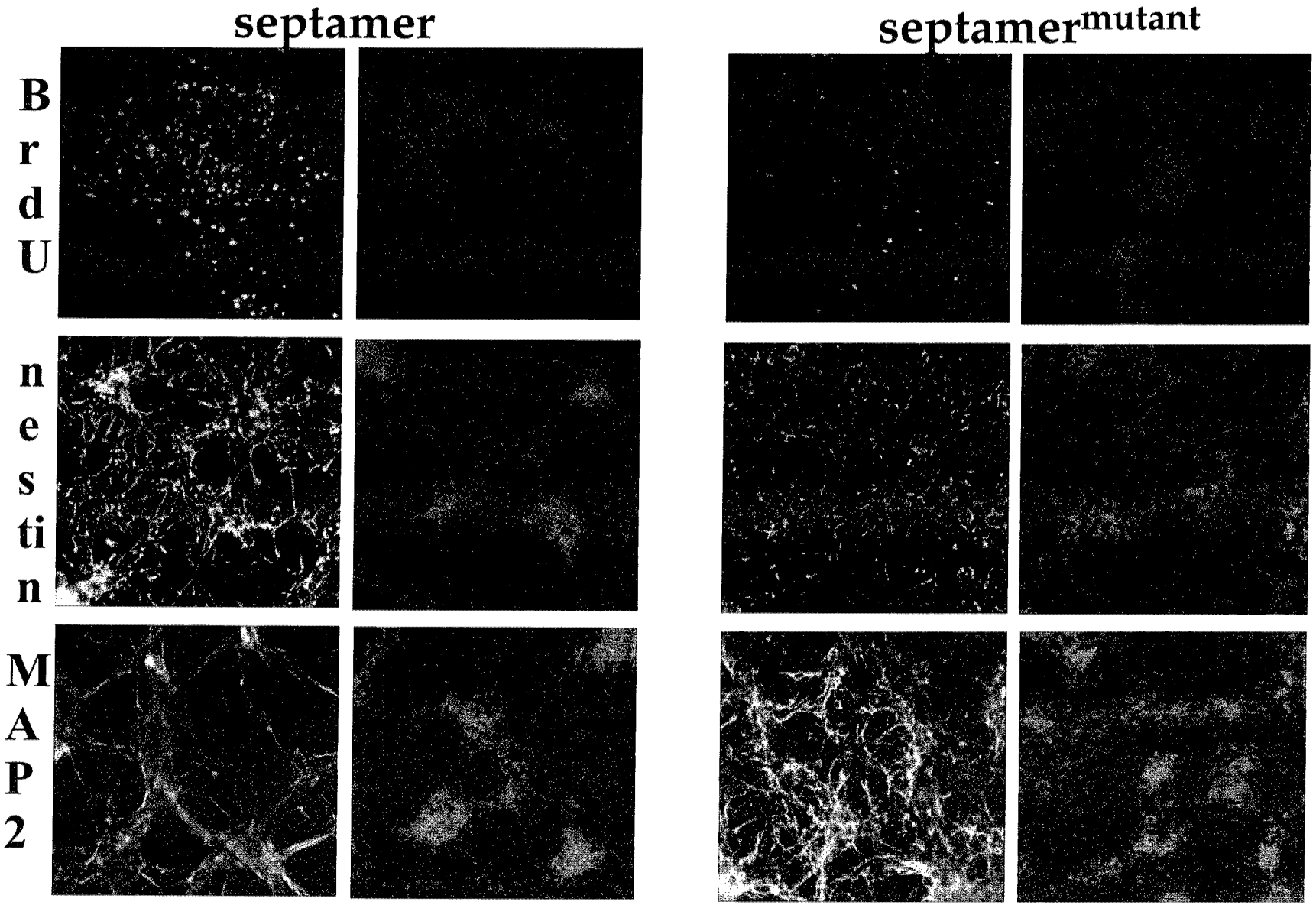
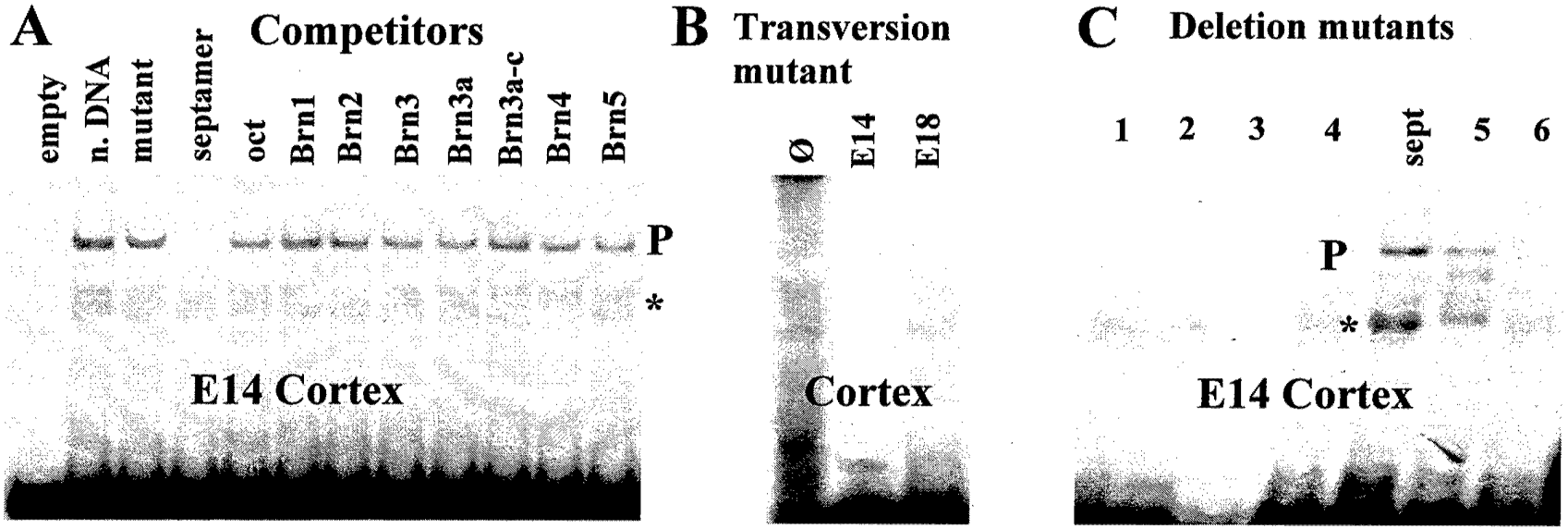



**Figure 1.** Septamer DNA molecular decoy treatment results in increased number proliferating, immature cells and decrease in mature neurons in cortical cultures. Cortical cultures derived from E16 rat brain were decoyed for 3 days using septamer or septamer mutant DNA. Cultures received BrdU ($10^{-5}$M), fixed and processed for BrdU, nestin and MAP2 immunohistochemistry using Cy3 as fluorochrome. Cellular nuclei were labeled with DAPI staining (blue).




**septamer**=TTCAAATATT GG**TTTGCAT**A ATCATTGACT GCCTACTGAG
**mutant**=TTCAAATATT GG**GGGTACG**A ATCATTGACT GCCTACTGAG

Deletion mutants used in C:

**1** AGTTTAAGATCTCCAGAAAGTTTCAAA
**2** GATCTCCAGAAAGTTTCAAATATTGG
**3** CCAGAAAGTTTCAAATATTGG**TTTGCAT**A
**4** AGTTTCAAATATTGG**TTTGCAT**AATCA
**sept** CAAATATTGG**TTTGCAT**AATCATTGAC
**5** TATTGG**TTTGCAT**AATCATTGACTGCC
**6** **TTTGCAT**AATCATTGACTGCCTAC

**Figure 2.** Specificity of septamer DNA decoy. Competitive EMSA using E14 cortex were performed in the presence of $^{32}$P-labeled septamer probe and its transversion mutant (T. mutant) or dsDNA representing the binding sites for various octamer/POU proteins as cold competitors used in **200-fold** excess. (**A**). The transversion mutant lacks septamer binding activity ( **B**). Left-to-right and right-to-left deletion mutant assay to determine minimum binding site and the role of flanking sequences in septamer binding specificity (Dobi et al., 1995). Probes used are listed above. empty=probe only; nDNA=neutral DNA; P=p-sept; star indicates transient, multimeric form of protein-DNA complexes observed also with various truncated probes ( **C**).

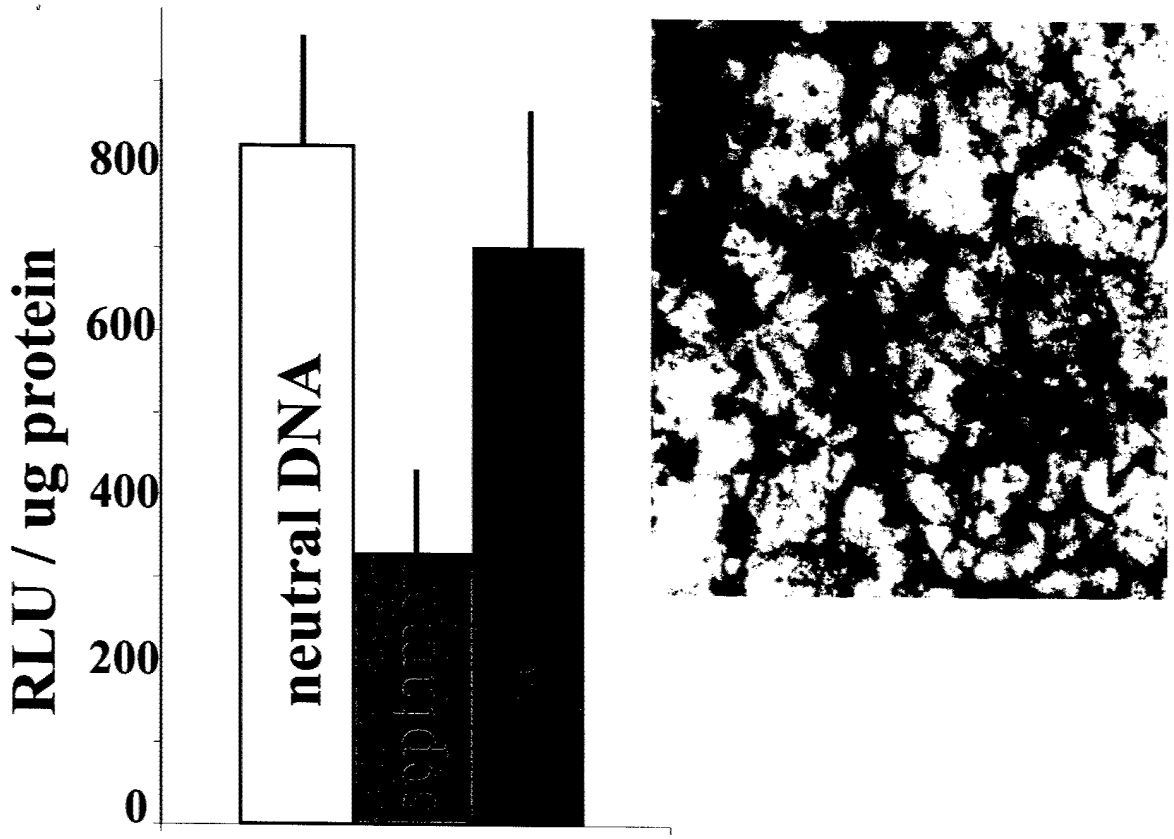



**Figure 3.** Testing septamer decoy molecules in a reporter gene system. Competitive transfection assay was performed using E16 cortical cultures. Cultures were -transfected with 0.5 ug of rENK-2700+703Luc, 2.0 ug of the competitor DNA (septamer, neutral or mutant) and 0.25 ug of the plasmid pCMV-Renilla Luciferase (Promega, Madison, WI) and carrier DNA up to 5 ug. DNA was mixed with 5 ul of 10 mM PEI in 100 ul of PBS (Boussif *et al.* 1995) and added to the culture medium. Firefly and renilla luciferase activities were measured by using Promega's Dual Luciferase Assay System. Relative light unit (RLU) represents values normalized for transfection efficiency.Values are mean +/- SEM. n=3. Insert illustrates the transfection efficiency of the PEI system using identical cortical cultures but CMV-B-gal plasmid. Three days after transfection cultures weere processed for X-gal histochemistry.